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A TH2-LIKE CYTOKINE RESPONSE IS INVOLVED IN BULLOUS PEMPHIGOID. THE ROLE OF IL-4 AND IL-5 IN THE PATHOGENESIS OF THE DISEASE.

C. FELICIANI, P. TOTO, S. MOHAMMAD POUR, G. COSCIONE, P. AMERIO[1] AND P. AMERIO[2]

Department of Dermatology, University "G.D'Annunzio", Chieti, Italy, and [1]Department of Dermatology, University of Ancona, Ancona, Italy.
[2]Department of Dermatology, Catholic University, Rome, Italy

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Bullous Pemphigoid is an autoimmune bullous disorder characterized by production of IgG against an hemidesmosomal antigen (230 kDa, 180 kDa) responsible for blistering of the skin. In the past several mediators have been implicated in the pathogenesis of the disease such as proteases and collagenases secreted by local inflammatory cells. In order to investigate the role of cytokines in BP, the cytokine pattern was evaluated by an immunohistochemical analysis and by reverse transcriptase polymerase chain reaction procedure in 13 BP patients. Cytokines examined were interleukin (IL)-2, IL-4, IL-5, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The T cell inflammatory infiltrate was also characterized by monoclonal antibodies showing CD3+, CD4+ T cells with a perivascular and scattered distribution in lesional skin. IL-4 and IL-5 were detected in a similar distribution to the inflammatory infiltrate. IL-4 and IL-5 mRNA levels were also revealed by RT-PCR. Proinflammatory cytokines such as TNF-α, IL-6 and Th1-like cytokines (IL-2 and INF-γ) were not detected neither as proteins nor as mRNA. Since IL-4 and IL-5 are important in eosinophil chemoattraction, maturation and functional activity, the presence of IL-4 and IL-5 in BP suggest that these cytokines could be important in the pathogenesis of the disease.

Bullous Pemphigoid (BP) is a subepidermal blistering disorder characterized by linear depositions of complement and IgG autoantibodies against the basement membrane zone (BMZ). Histological and immunohistological examination of BP lesions show T cells (mainly CD4+), neutrophils, eosinophils and mastcells (1-3). CD4+ T cells are divided into T helper1 (Th1) and a Th2 phenotypes. The Th1 phenotype produces IL-2, TNF-β and IFN-γ important in delayed hypersensitivity, the Th2 phenotype, which provides support to the humoral response, synthetizes IL-4, IL-5, IL-6, IL-10, IL13, and GM-CSF (4-6). Th2-like cytokines are important mediators in eosinophil activity, growth and maturation, they have been implicated in diseases where eosinophilia is prominent such as asthma (7) and atopic dermatitis (8). Studies involved in the comprehension and evaluation of the blistering process, in the disease, have progressively outlined the role of the inflammatory infiltrate (9-10). There are reports showing a Th1-like involvement in BP (11-13). Significant high levels of IFN-γ were found in serum and blister fluid of BP patients (11-13). IFN-γ has also been demonstrated to induce dermal-epidermal split, within the lamina lucida, in explanted cultured human skin (13). Elevated levels of IL-2R (14), TNF-α (15), TNF-β (16) and an IL-1 and IL-2 like activity (17) have been demonstrated in BP blister fluid or in the sera of BP patients. On the other hand several authors have shown a Th2-like involvement in the pathogenesis of the disease suggesting a potential role of IL-4, IL-6, IL-10 and IL-5 documented in blister fluid of BP patients

Key words: Bullous pemphigoid, cytokine, interleukin-4, interleukin-5, Th2

Mailing address: FELICIANI Claudio
Clinica Dermatologica
Via dei Vestini, 66013 Chieti, ITALY
Phone +39-0871-3556464, fax +39-0871-551057
e-mail fcliciani@unich.it

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(18-19). IL-4 and IL-5 are important cytokines in eosinophil chemoattraction and maturation (20-21). Eosinophils have assumed an important role in the pathogenesis of several skin diseases, including autoimmune bullous disorders (22). Eosinophil derived proteins, presumably proteases, seem to be directly involved in the dermal epidermal split (23) .

A prevalent Th2 type phenotype in other chronic subepidermal bullous diseases such as linear IgA dermatosis (24) and dermatitis herpetiformis (25) have been recently reported. This is the first report showing a Th2-like cytokine expression at a molecular level in BP and we suppose that IL-4 and IL-5 are important mediators in the development of the disease.

MATERIALS AND METHODS

Patients

Thirteen patients affected by BP were selected for this study (7 male, 6 female). Age ranged from 48 to 81 years (median age 65.8). Diagnosis was established according to clinical, histopathologic and immunologic (direct and indirect immunofluorescence on suction split-skin) criteria. All specimens were from patients with a recent onset of the disease and without any previous specific treatment. Skin specimens were immediately frozen in liquid nitrogen and stored at -80°C until use.

Immunohistochemistry

Cryostat sections were cut at 6(m and air dried for 30 minutes without fixation. Endogenous peroxidase was quenched in 0.3% H_2O_2. Single immunolabelling on serial sections was performed as previously described (26) using monoclonal mouse anti-human antibodies CD3, CD4, CD8, (YLEM, Rome, Italy), CD45RO (Dako, Glostrup, Denmark), TNF-α, IL-2, IL-4, IL-6, and IFN-γ; rabbit anti-human IL-5, IL-8 (Genzyme, Cambridge, USA), and TNF-α (Pepro-Tech, Rocky Hill, NJ, USA). Skin sections were incubated with primary antibodies, then incubated with biotinylated rabbit anti-mouse IgG or goat anti rabbit and finally reacted with HRP-streptavidin. In brief, primary antibodies were incubated at room temperature for 60-90 min in a moist chamber. For immunoperoxidase staining we used a streptoavidin-biotin system kit for primary mouse and rabbit antibodies (YLEM, Avezzano, Italy).

Diaminobenzidine tetrahydrochloride in PBS containing 0.01% fresh hydrogen peroxide was used as a chromogenic substrate. Sections were countstained with hematoxylin, dehydrated and permanently mounted with Eukitt (Kindler GmbH & co., Freiburg, Germany) under a coverslip.

Negative controls were performed using 1) normal skin, 2) omitting the incubation with primary antibodies. Cytokine expression were assessed as follows: strong staining, ++++/+++; moderate staining, ++; weak/focal staining, +; the epitelial staining was described as positive or negative.

mRNA extraction and cDNA synthesis

mRNA was extracted using a purification system kit (Pharmacia Biotech, Milan, Italy). In brief, about 30 of 20 μm cryostat sections from a 6 mm punch biopsy (about 0.5 gr of frozen tissue) were dissolved in a solution containing guanidinium thiocyanate 4M and N-lauroylsarcosine in order to preserve the RNA. The solution was placed in oligo(dt)-cellulose at 25 mg/ml suspended in a storage buffer containing 0.15% Kathon CG (Pharmacia LKB, Cologno Monzese (Ml), Italy). After several washes in salt buffers containing 10 mM Tris-HCl (PH 7.4), 1 mM EDTA, 0.5 M NaCl or 0.1 M NaCl in the last 2 washes, the oligo(dt)-cellulose containing mRNA was placed in filter columns and the mRNA was eluted in warm TRIS-HCl 10 mM and precipitated in chilled 95% ethanol overnight. After centrifugation, the pellet was dissolved in 14μl of DEPC-treated sterile water and quantitated by spectrophotometric analysis. 0.5 μg of mRNA was transcribed in cDNA incubating it with 200U of superscript reverse transcriptase (GIBCO BRL, Milan, Italy) and 50 ng of Random Examers.

Reverse Transcriptase-PCR

cDNA was amplified with 2.5 U Taq polymerase (Perkin Elmer Cetus, Milan, Italy) using 1.5 pM of each primer specific for IL-2, IL-4, IL-5, IFN-γ, β-actin. Primers were prepared from published sequences (27) using a DNA synthetizer (Applied Biosystem, Warrington, Great Britain). The sequences of specific primer pairs used in the PCR were as follows:
IL-2: upstream, 5'-ACTCACCAGGATGCTCACAT-3'; downstream 5'-AGGTAATCCATCTGTTCAGA-3'
IL-4: upstream, 5'-CTTCCCCCTCTGTTCTTCCT-3'; downstream, 5'-TTCCTGTCGAGCCGTTTCCAG-3'
IL-5: upstream, 5'-ATGAGGATGCTTCTGCATTTG-3'; downstream, 5'-TCAACTTTCTATTATCCACTCGGTGTTCATTAC-3'
IFN-γ: upstream 5'AGTTATATCTTGGCTTTTCA-3 '; downstream , 5 '-ACCGAATAATTAGTCAGCTT-3'

β-Actin: upstream, 5 '-GTGGGGCGCCCCAGG CAC-CA-3';
downstream, 5 '-CTCCTTAATGTCACGCACG ATTTC-3'

Each sample was divided in half, one part was used for the cytokine of interest, the other half for the house-keeping gene β-actin for semiquantitative analysis. RT-PCR was conducted for IL-4, IL-5, IL-2, IFN-γ and β-actin with the following protocol: 1 min. at 94°C, 1 min. at 58°C (62°C for IL-4 and β-actin), 1 min. at 72°. 30 cycles were used for cytokines of interest and 25 cycles for β-actin. The linear range of signal strength for each cytokine mRNA was determined by performing titration for cDNA and cycle numbers as previously described to obtain non saturated PCR reactions for each cytokine (28-29). Five μl of amplified products were electrophoretically separated in 2% agarose gel containing ethidium bromide and finally analyzed for molecular size. The following controls were used: 1) genomic DNA; 2) PCR products without primers; 3) normal skin (5 specimens). Signals were analysed by software Bio-profile (Vilber Lourmat, Nice, France), and semi-quantitative analysis was possible comparing the amplified product signals with the β-actin signal.

RESULTS

Clinical and immunophatological findings of BP patients are summarized in table 1. All patients demostrated depositions of IgG and C3 in the basement membrane and positive indirect immunofluorescence.

Table I also summarizes the immunohistochemical results and mRNA expression for each patient.

Immunohistochemistry

Immunohistochemical analysis showed a perivascular and scattered distribution of CD3+ T cells with a prevalent CD4+ T helper pattern with about 50% of CD45RO+ cells. CD45RA subset was also focally present.

Ten out of 13 patients affected by BP showed a positivity to IL-5 and IL-4. IL-4 and IL-5 staining showed a scattered and perivascular pattern in lesional (fig. 1a, 1c) and perilesional sites (fig.1b). Cells were mostly stained with an intracellular pattern, but some cells showed an extracellular staining. Rarely some intraepidermal cells were positive for IL-5 in BP (fig.1c). Controls and normal skin were negative. In lesional areas inflammatory cells consisting of CD4+ cell and

Case N.	Sex	Age	DIF	IIF	Cytokines staining	mRNA expression
BP1	M	75	IgG, C3	1:640	IL-4(+++), IL-5 (+++)	IL-4, IL-5
BP2	M	61	IgG, C3	1:320	IL-4(+++), IL-5(+++)	IL-4, IL-5
BP3	M	59	IgG, C3	1:160	IL-4(+), IL-5 (++)	IL-4, IL-5 (weak)
BP4	F	48	IgG, C3	1:320	IL-4(weak), IL-5(+++)	IL-4 (weak), IL-5
BP5	M	69	IgG, C3	1:160	-	IL-4, IL-5
BP6	M	50	IgG, C3	1:160	IL-4(+), IL-5(+++)	IL-4, IL-5
BP7	F	75	IgG, C3	1:160	IL-4(++), IL-5(+)	IL-4 (weak), IL-5
BP8	F	81	IgG, C3	1:160	-	IL-4, IL-5 (weak)
BP9	M	76	IgG, C3	1:320	IL-4(+), IL-5(+++)	IL-4 (weak), IL-5
BP10	F	67	IgG, C3	1:160	-	IL-4, IL-5
BP11	M	71	IgG, C3	1:640	IL-4(+++), IL-5(+++)	IL-4, IL-5
BP12	F	55	IgG, C3	1:320	IL-4(++), IL-5(+)	IL-4, IL-5 (weak)
BP13	F	69	IgG, C3	1:320	IL-4(+), IL-5(+++)	IL-4, IL-5

Table I. *Clinical and immunological characteristics of patients with BP:*
BP: Bullous Pemphigoid; DIF: Direct Immunofluorescence; IIF: Indirect immunofluorescence; -: Negative

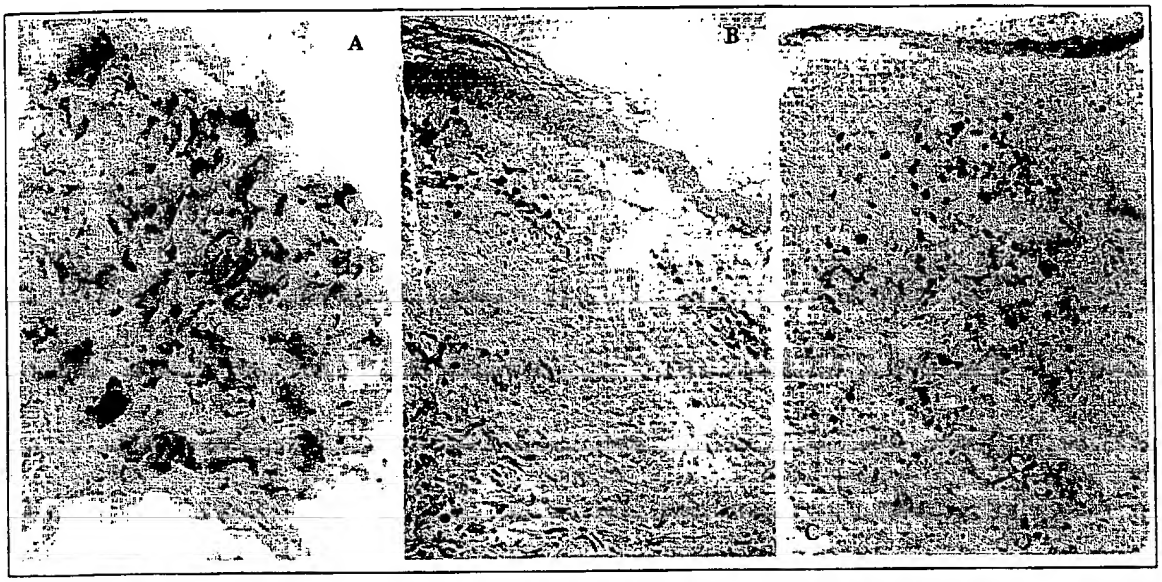

Fig. 1. *Immunohistochemical detection of IL-4 and IL-5 in BP: sections obtained from frozen biopsies were stained by the streptoavidin-biotin complex immunoperoxidase method (streptoABC-system) as described in Materials and Methods. A similar distribution of both proteins was seen. a) Presence of IL-4 protein in a blister lesionf of BP; b) pattern of IL-4 staining in perilesional skin; c) IL-5 protein in lesional skin BP, few positive cells are present in the epidermis.*

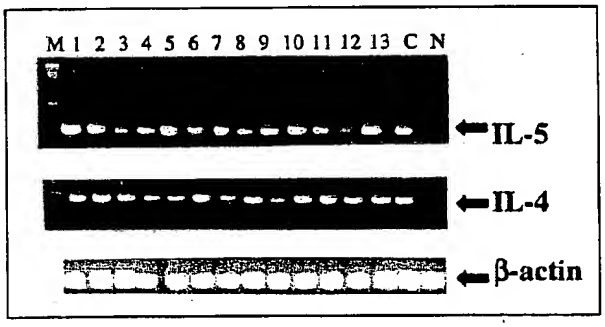

Fig. 2. *RT-PCR for IL-4 and IL-5 mRNA in Bullous Pemphigoid.*
Patients are numbered from 1 to 13. M=Marker; C= Positive Control; N= Normal skin.

eosinophils roughly corrisponded to the IL-4 and IL-5 staining. IFN-γ, IL-2 and TNF-α were under detectable levels.

Th2-like cytokines mRNA expression

As shown in figure 2 all BP patients expressed IL-4 and IL-5 mRNA. IL-4 or IL-5 expression appears not to be related to the disease activity. Most of the patients showed a similar expression between IL-4 and IL-5 (eg patients 1,2,4,10,11,13), others showed an higher IL-4 expression (eg. patients 3,6,8,12) and only two (patients 5 and 7) showed an higher IL-5 expression. Controls failed to express both IL-4 or IL-5 even after 50 PCR-cycles. No signals were detected for IL-2 after 60 cycles, IFN-γ was evident after more than 50 cycles both in normal and diseased skin in accordance with previously published results (27).

DISCUSSION

Bullous pemphigoid (BP) is an autoimmune blistering disease, characterized by linear IgG (mainly IgG4) and C3 deposits at the dermal-epidermal basement membrane (BMZ). A pathogenic role for specific antibodies has been demonstrated both in vitro on skin esplants (30) and in vivo using a passive transfer model in mice (31). Neverthless the presence of activated T cells within BP lesions and in the peripheral blood of patients, accompanied by eosinophilia, high levels of eosinophilic cationic

protein (ECP) and IgE, indicates the involvement of a cell mediated immune reaction in the process of blistering (32,33, 7, 10).

The T-cell component in BP displays a predominant T Helper pattern (CD4:CD8=2:1), contrasting data, however, regard the Th1 vs Th2 polarization of this infiltrate (1,2,7,34). Kaneko et al. report the presence of Th1 lymphocytes producing IFN-((12). Consistent with this finding IFN-(has been shown to induce, in vitro, a dermal epidermal split within the lamina lucida (11, 13). More recently, however, a Th2-like activity has been demonstrated in BP. The finding of increased serum CD30 levels, a specific activation marker of cells able to produce Th2 cytokines (35), in BP patients, seems to suggest that these cytokines play a role in the disease (36). In the same study, De Pita and coll. found a correlation between sCD30 and IL-4 serum levels (36). However, more direct evidence for a role of Th2 cytokines in the process of blistering comes from studies showing a higher level of these mediators in BP blister fluids than in corresponding serum samples (37-39). Previous reports found IL-4 and IL-5 in BP blister fluid by ELISA method (37-40). In the present study we confirm, by immunohistochemistry, the presence of IL-4 and IL-5 protein within BP lesions, and we demostrate IL-4 and IL-5 mRNA expression in BP lesions. Although double staining with anti IL-4 and anti CD4 or with anti IL-5 and CD4 was not feasible, it is very likely that IL-4 and IL-5 found in the skin could be produced and expressed by by CD4+ cells and eosinophils. Infact most of the cellular infiltrate in BP is represented by eosinophils, neutrophils and T cells, though mast cells play a role as well (1,3,9). The majority of this T cell infiltrate has the characteristics of the activation state (expression of HLA-DR antigens, production of IL-2, association with CD1a+ positive cells) (2,10,14). In our study the finding of high levels of IL-4 and IL-5 both messenger and protein and almost undetectable messenger for IFN-γ clearly indicates that T cells are polarized towards a TH2 phenotype, as we have already shown in HD, another autoimmune subepidermal disease with tissue eosinophilia (25). Since we describe a local production of IL-4 and IL-5 in the site of blistering we think that these cytokines could play a central role in this process. We speculate that the binding of the autoantibody to the target antigen activates the complement thus producing an inflammatory infiltrate. Chemoattracted Th2 cells, producing IL-4 and IL-5 could induce eosinophil activation. IL-4 exerts pleiotropic effects by acting on eosinophil recruitment in tissues (41). It promotes eosinophil transmigration from vascular endothelium by upregulating important adhesion molecules such as ICAM1 (42) and VCAM1 (43). This last one has been detected on vessels in blistering areas of BP in association with E-selectin and P-selectin (44). IL-5 shares its properties on eosinophils with IL-4 although IL-4 seems to act mainly on growth (21), while IL-5 has effects on eosinophil chemoattraction and activation in vitro and in vivo (45-48). Experimental data indicate that the accumulation of eosinophils within BP lesions is directly related to blistering process (44). Infact eosinophil granule proteins, though represented in all stages of BP lesions, are most marked in early erythematous and prebullous (urticarial) areas and is minimal in uninvolved skin (44). Furthermore it has been demonstrated, in vitro, that 92 KD gelatinase, a protease expressed only by eosinophils in BP lesions, has the ability to cleave the extracellular collagenous domain of recombinant 180 kDa BP autoantigen, thus contributing significantly to the dermal epidermal split in BP (23).

Taken together, these data provide further support to the hypothesis that the infiltrating T cells in BP are stimulated immunologically, showing a preferential polarization toward Th2-like phenotype. We also speculate that IL-4 and IL-5 could play a central role in recruiting, in situ, polymorphonuclear cells able to release factors capable of detaching the basal membrane. In BP patients an immunologic response might be present which involves not only the production of IgG autoantibodies directed against specific cutaneous antigens but also the activation of T cells, polarized into a Th2-type profile. Th2 cells, producing IL-4 and IL-5, could induce eosinophil activation. Eosinophils, together with mast cells and neutrophils, in this environment, could produce proteases responsible for the proteolytic action on the basement membrane.

In conclusion our report supports the hypothesis that Th2-like cytokines IL-4 and IL-5, in association with other mediators, are important in mediating the local immune response in BP.

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Spontaneous B-cell IgE production in a patient with remarkable eosinophilia and hyper IgE

Shuji Takashi, MD; Yoshio Okubo, MD; Shiro Horie, MD; Tomoyasu Momose, MD; Akihiro Tsukadaira, MD; Kazunaga Agematsu*, MD; and Morie Sekiguchi, MD

Background: The pathophysiology of eosinophilia and hyper-IgE is not fully elucidated yet.

Objective: To clarify the pathophysiology of a patient with remarkable eosinophilia and hyper IgE, we examined cytokine levels in serum, surface antigens of peripheral blood eosinophils and IgE production in vitro.

Results: Concentrations of tumor necrosis factor-α (TNF-α), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), and granulocyte/macrophage-colony stimulating factor (GM-CSF) in the serum were 21 pg/mL, <15 pg/mL, <15 pg/mL, 8 pg/mL, and <5 pg/mL pg/mL, respectively. Newly expressed surface antigens CD4, CD25, CD69, and HLA-DR, but not CD54, were observed on peripheral blood eosinophils. Extremely high levels of IgE secretion was found in the patient's mononuclear cells without stimuli; this was not enhanced by IL-4 or IL-4 plus anti-CD40 monoclonal antibody stimulation. Furthermore, highly purified B cells spontaneously produced large amounts of IgE and the production was not enhanced in addition of his T cells.

Conclusion: The eosinophils were activated, and the B cells spontaneously produced IgE independently of T cells or cytokines, suggesting that intrinsic abnormality of B cells leading to disregulated production of IgE in this disease.

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INTRODUCTION

The IgE immune response is important because it leads to the development of allergic diseases such as rhinitis and bronchial asthma.[1] The production of immunoglobulin E (IgE) from B cells requires at least two signals: a class switching factor, most commonly interleukin (IL)-4, and the engagement of CD40 molecule on B cells. The combination of IL-4 and anti-CD40 monoclonal antibody (mAb) promotes IgE production in vitro.[2–4]

Eosinophilia is reported in various diseases such as bronchial asthma, parasite infection and Churg-Strauss syndrome. Mature eosinophils are developed in the presence of interleukin-3 (IL-3), interleukin-5 (IL-5), and granulocyte/macrophage-colony stimulating factor (GM-CSF).[5] The functions of eosinophils are survival elongation in vitro,[6] degranulation,[7] release of chemical mediator[5] and cytokine production.[8] Surface antigens on eosinophils are expressed by various cytokine stimulation[9] and the newly expressed surface antigens are suggested to be a hallmark of activation.[10,11]

To clarify the pathophysiology of a patient with high levels of serum IgE and remarkable eosinophilia, we have examined the concentration of cytokines in serum, surface analysis of eosinophils and IgE synthesis in vitro.

CASE REPORT

A 82-year-old man was transferred to our hospital because of edema on the extremities, systemic erythema, eosinophilia, and high levels of serum IgE. He was treated twice for tuberculous pleurisy (16 years old and 26 years old). He had suffered from atopic dermatitis for 5 years and was treated with steroid ointment. In August 1997, he was admitted to a hospital because of pyrexia. A diagnosis of pulmonary tuberculosis was made based on positive acid-fast stain of the sputum and consolidation shadow of right middle-lower lung fields on chest roentogenogram; the patient was administered the anti-tuberculous drugs isoniazid, rifampicin and ethambutol. Although liver dysfunction occurred (GOT 500 U/L, GPT 487 U/L, LDH 1,092 U/L), it was improved by quitting administration of the anti-tuberculous drugs. The number of peripheral blood eosinophils, however, gradually increased (2,000 to 8,000/μL), and systemic erythema worsened in conjunction with the appearance of edema in the lower extremities. On November 19, 1997 he was transferred to our hospital for further examination. His temperature was 37.6°C; several 5 to 10 mm sized superficial lymph nodes were palpable on the bilateral neck, left axilla and bilateral groins. Liver was palpable 3 cm on right midclavicular line. White blood cells of peripheral blood (15,700/μL), eosinophils (8,007/μL) and LDH (813 U/L) were elevated. Serum IgE and CRP were 25,207 IU/mL and 1.09 mg/dL, respectively. Drug-induced lymphocyte stimulation test for antituberculous drugs was negative. Repeated tests for parasites in the feces were negative. Chest roentogenogram showed consolidation on right middle-lower lung fields with pleural calcification. Biopsy and aspiration of bone marrow showed increased cell number of mature eosinophils without malignant cells and a lymph node biopsy specimen taken from the right inguinal region showed no malignancy. Steroid ointment was

From First Department of Internal Medicine and *Department of Pediatrics, Shinshu University, School of Medicine, Matsumoto, Japan.

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administered; edema on extremities and systemic erythema improved and peripheral eosinophil count gradually decreased (500 to 3,000/μL), but the high levels of serum IgE persisted. The patient was discharged on February 11, 1998 with high levels of serum IgE (133,870 IU/mL).

MATERIALS AND METHODS

Antibodies and Reagents

FITC-conjugated mAbs against CD9 (mouse IgG1, Pharmingen, San Diego, USA), CD20 (mouse IgG1, Dakopatts A/S, Denmark), CD25 (mouse IgG1, Dakopatts A/S), CD54 (mouse IgG1, Immunotech SA, Marseille, France), CD69 (mouse IgG1, Becton Dickinson, San Jose, USA), VLA-4 (mouse IgG1, Immunotech SA) and phycoerythrin (PE)-conjugated mAbs against HLA-DR (mouse IgG1, Becton Dickinson), CD4 (mouse IgG1, Becton Dickinson), isotype-matched FITC- or PE-conjugated mouse IgG1 mAbs (Dako patts A/S), purified anti-CD40 mAb (IgG1, Immunotech SA), recombinant human (rh) IL-4 (Genzyme Corp, Boston, USA) were purchased.

Cell Preparation

Peripheral blood mononuclear cells (PBMNCs) from the patient and normal subjects were isolated by Ficoll-Hypaque (Pharmacia, NJ, USA) density gradient centrifugation. Furthermore, erythrocyte rosette-positive (E+) and negative (E−) populations[12] were separated with 5% sheep erythrocytes. After depleting monocytes with silica (IBL, Fujioka, Japan) or adherence to the plastic surface, E− cells were further purified into B cells by positive selection with anti-CD19 mAb-coated immunomagnetic beads (Dynal, Oslo, Norway); anti-CD19 mAb was then removed by use of Detach a bead (Dynal). Ninety-seven percent of B cells were reactive with anti-CD20 mAbs. The B cells thus obtained had some tyrosine phosphorylations, whereas no proliferation and activation were found.

Cytokine and Chemokine Measurements

Peripheral blood (PB) was taken from the patient before steroid treatment. Peripheral blood was kept at room temperature for 30 minutes. Serum for cytokine measurement was obtained by centrifugation at 400 *g* for 10 minutes at 4°C. These samples were kept at −70°C until assay. Cytokine concentration was measured using an ELISA kit. The kits for tumor necrosis factor-α (TNF-α) (Medgenix Diagnostics, Fleurus, Belgium), interleukin-3 (IL-3) (Amersham International plc. Buckinghamshire, UK), interleukin-4 (IL-4) (Amersham International plc.) and interleukin-5 (IL-5) (R&D System, Minneapolis, USA), granulocyte/macrophage colony stimulating factor (GM-CSF) (Amersham International plc.), macrophage/monocyte chemotactic protein-3 (MCP)-3 (Toray Co, Tokyo, Japan) and regulated on activation, normal T cell expressed and secreted (RANTES) (Amersham International plc.) were employed. The minimum measurable amounts of TNF-α, IL-3, IL-4, IL-5 and GM-CSF are 6 pg/mL, 15 pg/mL, 15 pg/mL, 8 pg/mL, 5 pg/mL, 200 pg/mL and 50 pg/mL, respectively. Each cytokine level of normal control serum was below the minimum measurable sensitivity.

Surface Analysis

To analyze surface antigens of eosinophils, we purified PB eosinophils using an improved immunomagnetic selection procedure.[13] Briefly, 20 mL heparinized venous peripheral blood was mixed with piperazine-N, N-bi (2-ethane sulfonic acid) (PIPES) buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, PH 7.4) at a 1:1 ratio. The diluted blood was overlayered on isotonic Percoll solution (1.082 g/mL) (Sigma) and centrifuged at 1,000 *g* for 30 minutes at 4°C. The mononuclear cell layer was then removed and erythrocytes in the sediment were lysed using two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with PIPES buffer with 1% inactivated fetal calf serum (FCS) (Life Technologies, Inc, Gaithersburg, USA). The pellet of granulocytes was incubated with anti-CD16 mAb coated immunomagnetic particles (Miltenyi Biotec, Bergish-Gladbach, Germany; 50 μL for 5×10^7 cells) for 60 minutes at 4°C. Magnetically labeled neutrophils were then depleted by passing the granulocytes through a magnetic cell separation (MACS) column in the field of a permanent magnet (Miltenyi Biotec). This procedure resulted in more than 98% pure eosinophils with less than 2% contamination by neutrophils and without lymphocytes; viability was more than 98%. The purity and viability of eosinophils was established using Randolf staining and trypan blue dye exclusion, respectively. Ten or twenty microlitres of FITC-conjugated mAbs against CD9, CD25, CD54, CD69, VLA-4 and phycoerythrin (PE)-conjugated mAbs against HLA-DR, CD4 and isotype-matched FITC- or PE-conjugated mouse IgG1 mAbs were reacted with PB eosinophils (5×10^5/50 μL) for 30 minutes at 4°C. Peripheral blood eosinophils were washed twice with PBS containing 1% inactivated FCS at 300 *g* for 10 minutes. The cells were resuspended in PBS containing 1% inactivated FCS. Gated area by forward light scattering and side light scattering confirmed the presence of eosinophils using anti-CD9 and anti-VLA-4 mAbs by flow cytometry (FACScan, Becton Dickinson). Surface analysis was then performed as reported previously.[13]

Immunoglobulin Assay by ELISA

Peripheral blood mononuclear cells were cultured with 50 ng/mL of IL-4 and 1 μg/mL of anti-CD40 mAb at a final cell density of 0.125 to 2×10^6/mL in a volume of 200 μL/well for 12 to 14 days at 37°C in a humidified atmosphere with 5% CO_2. Highly purified peripheral blood B cells (0.5 to 2×10^5/mL) were also cultured with or without his E+ cells (50% of purified B cells, 0.25 to 1×10^5/mL). The cultured supernatants were harvested, and the supernatants and the standard human IgE (Chemicon International

Inc, Temecula, CA, USA) were added to human IgE mAbs (CIA-E-7.12 and CIA-E-4.15, kindly provided by A. Saxon, Division of Clinical Immunology/Allergy, UCLA School of Medicine, Los Angeles, CA) and applied to 96 well flat ELISA plates (Nunc, Roskilde, Denmark). After overnight culture at 4°C, the supernatants were discarded and the wells were washed with 0.05% tween 20 in phosphate buffered saline (PBS); alkaline phosphatase-labeled goat anti-human IgE (Sigma Chemical Co, St. Louis, MO) was then added at a dilution of 1/5,000. After 2 hours incubation at room temperature, color detection was performed using 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer containing *p*-nitrophenyl phosphate (pNPP) (Sigma). Calibration was performed with PBS at standard zero levels. All experiments were performed in duplicate. The minimum measurable amount of IgE is 8 ng/mL.[14,15]

RESULTS

Cytokine and Chemokine Measurements

To clarify the mechanism of the eosinophilia and extremely high levels of serum IgE, we initially examined serum concentrations of several cytokines which affect eosinophilia or IgE synthesis. The level of TNF-α in serum was 21 pg/mL, while IL-3, IL-4, IL-5, and GM-CSF could not be detected. The levels of MCP-3 and RANTES were 0.8 ng/mL and 28.5 ng/mL, respectively. These levels were within the range of normal subjects (MCP-3: 1.1 ± 0.5 ng/mL; RANTES 27.8 ± 4.6 ng/mL).

Surface Analysis of Eosinophils

To confirm the presence of eosinophils antibodies against CD9 and VLA-4 were used for cytometric analysis. CD9 and VLA-4 were expressed on the cells in the gated area, showing these cells were eosinophils (Fig 1a). It was reported previously that CD4, CD25, HLA-DR, CD54 and CD69 do not express on eosinophils of normal subjects. As shown in Figures 1a and b, the profiles of CD25 (30%), CD69 (13%), CD4 (13%), and HLA-DR (35%), but not CD54 were significantly different from those of isotype matched control, demonstrating them to be newly expressed antigens on the eosinophils. CD25, CD69, CD4, CD54 and HLA-DR on a normal subject's eosinophils were not detected in parallel analysis.

Spontaneous IgE Production

Since the patient's serum contained extremely high levels of IgE, we thoroughly investigated IgE production. As shown in Figure 2, PBMNCs obtained from the patient produced high levels of IgE in a cell number dependent fashion without any stimulation. In contrast, PBMNCs obtained from normal subjects produced low levels of IgE with IL-4 plus anti-CD40 mAb stimulation (n = 10), whereas no IgE production (<8 ng/mL) was recognized in medium alone or IL-4 alone (Fig 2). Interestingly, the addition of IL-4 or IL-4 plus anti-CD40 mAb did not enhance IgE secretion compared with medium alone in the patient, indicating spontaneous production of IgE by his PBMNCs. We proceeded to examine IgE production by using highly purified B cells from the patient's peripheral blood. As shown in Figure 3, highly purified B cells produced high levels of IgE in a cell number dependent fashion; this production was not affected by the addition of IL-4 or IL-4 plus anti-CD40 mAb (data not shown). Furthermore, IgE production was not enhanced by the addition of T cells obtained from the patient into purified B cells. Purified B cells obtained from normal subjects showed low levels of IgE production with IL-4 plus CD40 signaling (purified B cells 1×10^4/well: 10 ± 2 ng/mL; 2×10^4/well: 16 ± 7 ng/mL; 4×10^4/well: 21 ± 9 ng/mL, n = 5), but no IgE production with medium alone (Fig 3) or IL-4 alone (data not shown). These data demonstrate that B cells from the patient spontaneously produced IgE.

DISCUSSION

Hyper-IgE syndrome is characterized by an immunodeficiency disease which causes frequent staphylococcal abscesses, persistent eczematoid rashes, and extreme elevations of IgE in serum. Several observations such as erythema, positive acid fast staining, eosinophilia, high level of IgE in serum of the patient were similar to previous reports.[16,17] Recently, it was reported that hyper-IgE syndrome is an autosomal dominant multisystem disorder,[17] however the patient's family shows no features of the hyper-IgE syndrome.

Eosinophilia is observed in various diseases such as bronchial asthma, helminthic infection,[18] hyper-IgE syndrome[16,17] and in IL-2 administration.[19] Interleukin-3, IL-5, and GM-CSF are active in stimulating eosinophilopoiesis.[5] Interleukin-5 transgenic mice have developed massive, life-long eosinophilia,[20] suggesting IL-5 would be involved in a candidate mechanism for explaining hypereosinophilia; however, these cytokines were not detected in the patient's serum. The mechanisms of eosinophilia were not clear in this patient, suggesting other eosinophilic factor(s) were involved.

It is reported that numerous surface antigens are constitutively expressed on human eosinophils.[5] Hypodense eosinophils exhibit various morphologic changes such as cytoplasmic vacuolization, alterations in granules of major basic protein-containing cores or matrix.[18] These morphologic changes are well correlated with activated eosinophils induced by cytokines' (IL-3, IL-5 and GM-CSF) stimulation in vitro. There are several reports concerning newly expressed antigens in a variety of diseases. Expression of CD4 and CD25 on PB eosinophils was reported in hypereosinophilic syndrome (HES), helminth infection, and melanoma or renal cell carcinoma treated with IL-2.[19,21] CD69 expression on PB and bronchoalveolar lavage fluid (BALF) eosinophils was reported in HES and helminth infection.[22] These findings suggest that activated heterogenous eosinophils are present in various eosinophilic disorders. In this case, CD4, CD25, CD69 and HLA-DR, but not CD54, are expressed on eosinophils.

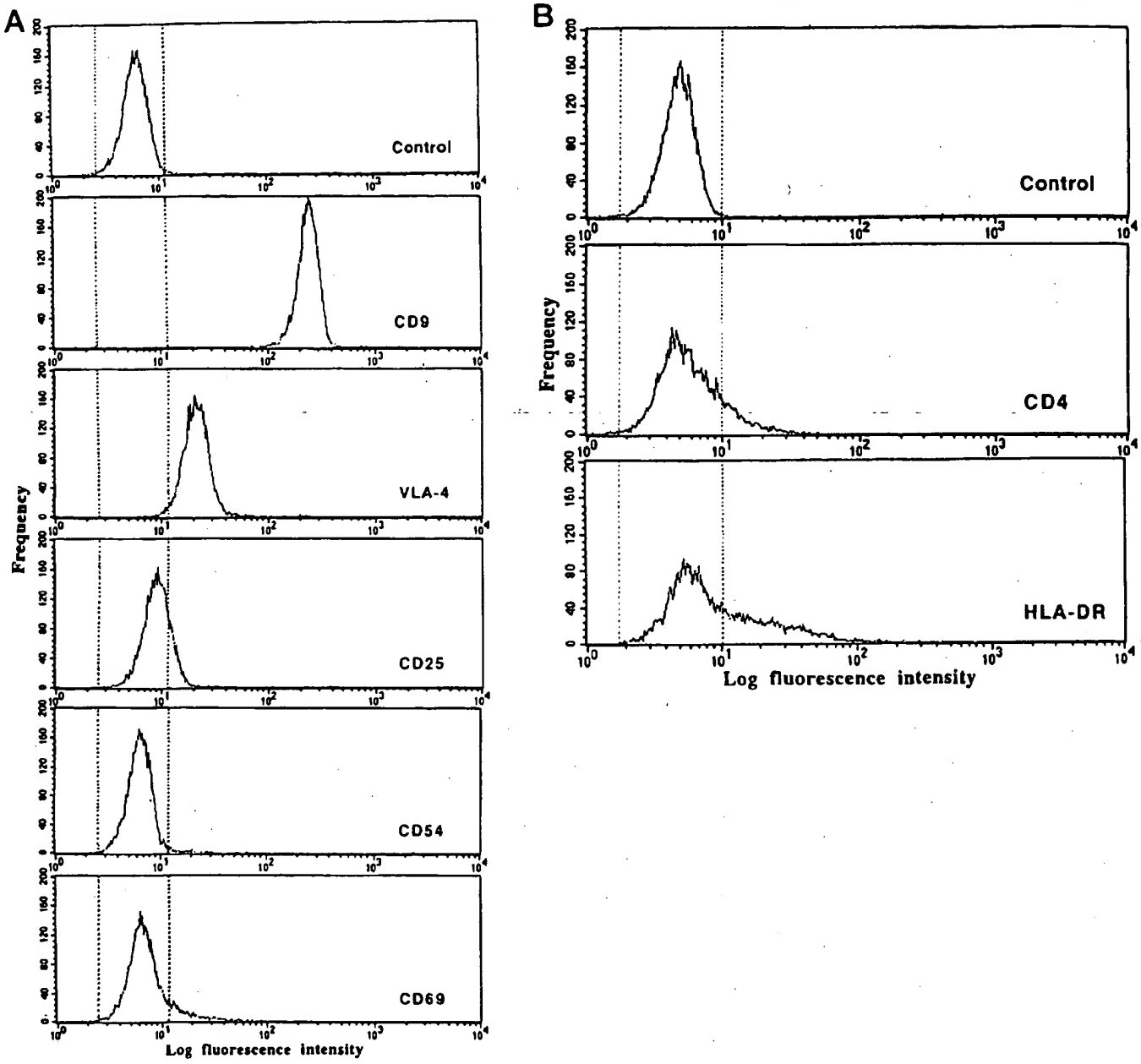

Figure 1. Surface analysis of peripheral blood eosinophils using flow cytometry. Newly expressed surface antigens (CD25 and CD69), but not CD54 could be detected (Fig 1a). Newly expressed surface antigens (CD4 and HLA-DR) could be detected (Fig 1b).

The reason is unclear, however the interaction of cytokines and chemokines may be involved in expression of eosinophil surface antigens.

IgE production of B cells is regulated by cytokines such as IL-4,[3,23,24] IL-13,[25,26] and direct contact between B and helper T cells,[2,24,27,28] in which CD40/CD154 interaction plays a key role in IgE synthesis.[3] Although it is known that IL-4 has a potent function in the production of IgE,[29,30] IL-4 alone induced germline ε transcripts, but not IgE production. Since our patient's B cells produced IgE spontaneously, we investigated germline ε transcripts using the patient's E-cells without stimuli; however, germline ε transcripts were not observed (data not shown). There is a possibility that, to our regret, we could not obtain enough B cells. EB virus-transformed B cell lines established from the patient also produced IgE spontaneously at first, but

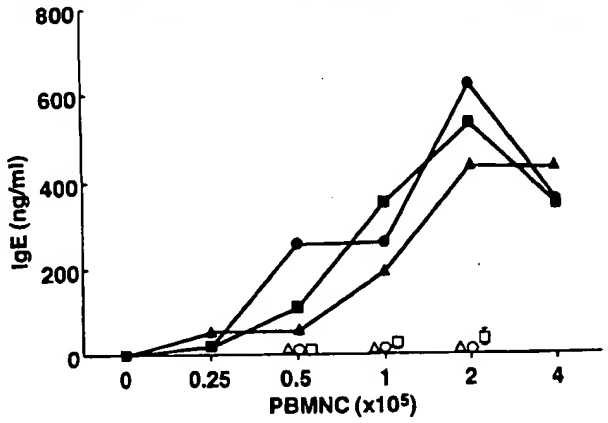

Figure 2. IgE production by peripheral blood mononuclear cells (PBMNCs) of the patient and normal subjects (n = 10). Peripheral blood mononuclear cells were cultured with medium (▲: patient and △: normal subjects), IL-4 (●: patient and ○: normal subjects), or IL-4 plus anti-CD40 mAb (■: patient and □: normal subjects) at various concentrations in 96-well round-bottom plates for 12 to 14 days. IgE contents were measured using ELISA. Results are expressed as the mean ± SE of triplicates, and error bars are sometimes smaller than the plot symbol.

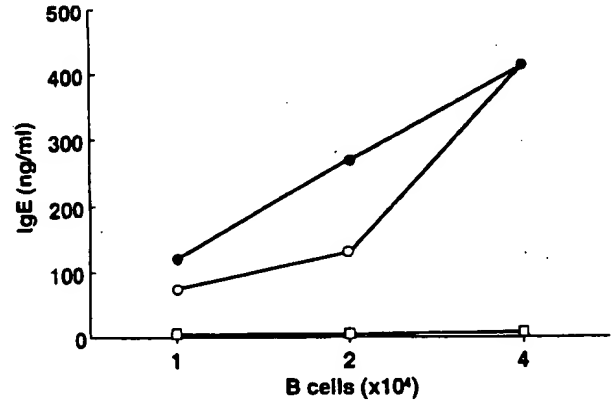

Figure 3. IgE production by purified B cells of the patient and normal subjects (n = 5). Highly purified B cells were cultured with medium alone (○: patient and □: normal subjects) and patient's purified B cells were also cultured with autologous patient's T cells (●) at various concentrations in 96-well round-bottom plates for 12 to 14 days. IgE contents were measured using ELISA. Results are expressed as the mean ± SE of triplicates.

not EB virus-transformed B cell lines obtained from normal volunteers (n = 5), whereas the IgE production was gradually decreased and disappeared according to the culture (data not shown). Other immunologic studies of hyper-IgE syndrome have reported little responsiveness of exogenous IL-4 for IgE production.[31] Claassen et al reported neither PBMNCs nor B cells from the patients with hyper-IgE syndrome spontaneously produce IgE in vitro, little responsiveness of exogenous IL-4 for IgE production and requirement of high concentration of anti-CD40 for IgE production.[31] Our data presented here are clearly different from the report by Claassen et al in the point of spontaneous IgE production of PBMNCs and B cells and no response of anti-CD40 for IgE production, however, it is similar to the data to be little responsiveness of exogenous IL-4 for IgE production. It is considered that once B cells become committed to IgE synthesis, IL-4 appears to have little role in the regulation of IgE production. Another possibility in low responsiveness of IL-4 in IgE production is due to variant IL-4 receptor.[32] We did not examine the variant IL-4 receptor in the patient. Grimbacher et al reported that IL-4 receptor variant allele was not significantly different from control subjects, suggesting IL-4 receptor gene is not linked to the hyper-IgE syndrome.[32]

In conclusion, we report a patient with remarkable eosinophilia and hyper-IgE. Although several cytokines were not detected in the serum, the eosinophils in peripheral blood were activated and the B cells spontaneously produce large amounts of IgE in vitro. Further analyses about the patient are needed to clarify mechanisms of eosinophil activation and IgE production, which will contribute the treatment of allergic diseases.

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Request for reprints should be addressed to:
Yoshio Okubo, MD
First Department of Internal Medicine
Shinshu University School of Medicine
3-1-1 Asahi
Matsumoto 390-8621
Japan

Requirements for αd in IgG Immune Complex-Induced Rat Lung Injury[1,2]

Thomas P. Shanley,* Roscoe L. Warner,* Larry D. Crouch,* Gregory N. Dietsch,† Darcey L. Clark,† Maggie M. O'Brien,† W. Michael Gallatin,† and Peter A. Ward[3]*

αd is a newly cloned adhesion molecule that forms a heterodimer with CD18. The requirement for αd in IgG immune complex-induced lung injury in rats has been evaluated by the use of blocking polyclonal and monoclonal antibodies to rat αd. Using whole lung extracts, Northern and Western blot analyses have revealed up-regulation of mRNA and αd protein in inflamed lungs. Immunostaining has revealed the presence of αd in lung tissue and in alveolar macrophages as early as 1 h after initiation of the inflammatory reaction. When polyclonal rabbit Ab to rat αd was coinstilled into lung together with Ab to BSA, lung injury (as determined by leakage of [^{125}I]albumin into lung parenchyma) was significantly diminished. In parallel, there was reduced accumulation of neutrophils recoverable in bronchoalveolar lavage (BAL) fluids. These findings were associated with reduced levels of TNF-α as well as NO_2^-/NO_3^- in BAL fluids. A hamster mAb to rat αd was also protective in this lung injury model. Anti-αd inhibited in vitro production of NO_2^-/NO_3^- by rat alveolar macrophages (stimulated with LPS and IFN-γ) by approximately 60%. These data suggest that, in the lung inflammatory model employed, αd up-regulation occurs in lung macrophages and is necessary for expression of TNF-α, recruitment of neutrophils, and full development of lung injury. *The Journal of Immunology,* **1998, 160: 1014–1020.**

Intercellular adhesion molecule-3 (ICAM-3),[3] a member of the Ig supergene family, has recently been cloned (1–3) and is a known "counter-receptor" for CD11a/CD18, the molecular interaction of which appears to facilitate T cell responses to Ags (4–9). ICAM-3 is expressed on "nonstimulated" human neutrophils, monocytes, and T cells, as well as on epidermal dendritic cells and Langerhan cells in the skin (10–17). In abnormal tissues, ICAM-3 has been found expressed on lymphoma cells (18), on endothelial cells within breast tumors of humans (19), on infiltrating cells in renal allografts, in mesangial leukocytes in patients with membranous glomerulonephritis (20), and in rheumatoid synovial tissue (21). Recently, human αd has been cloned and, as part of the heterodimeric complex, αd/CD18, it appears to be a counter-receptor for ICAM-3 (22), although the in vivo role of αd/CD18 is not known.

In models of acute lung injury in rats, the recruitment of neutrophils from the intravascular space to the alveolar compartment has been the focus of recent investigations. Depending on the inflammatory model, this process has been shown to be dependent on engagement of a series of endothelial cell adhesion molecules (e.g., E-selectin, P-selectin, ICAM-1, PECAM-1 (platelet endothelial cell adhesion molecule)) as well as neutrophil adhesion molecules (L-selectin, β_2-integrins) (23–26). The recent cloning and expression of rat αd has allowed us to explore the role of this novel β_2-integrin in the setting of the acute lung inflammatory response triggered by deposition of IgG immune complexes. Up-regulation of αd at both the mRNA level (as determined by Northern blot analysis) and protein level (as demonstrated by Western blot analysis and immunohistochemical staining) was found. Blocking of αd function with Abs resulted in diminished inflammatory responses, as measured by pulmonary vascular leak and content of neutrophils in bronchoalveolar lavage (BAL) fluids. Furthermore, in vivo blocking of αd appeared to result in decreased activation of lung macrophages, as evidenced by substantial decreases in both TNF-α and NO_2^-/NO_3^- content. These data suggest that αd plays a critical role in the activation of macrophages in the context of IgG immune complex-initiated lung injury in rats.

Materials and Methods

Chemicals and reagents

Except where noted, all products were purchased from Sigma Chemical Co., (St. Louis, MO).

IgG immune complex-induced alveolitis

Male Long-Evans (specific, pathogen-free) rats (250 to 350 g, Charles River Breeding Laboratories, Portage, MI) were used for all studies. Intraperitoneal injections of ketamine (2.5 to 5.0 mg/100 g body weight) were given for sedation and anesthesia. IgG immune complex lung injury was induced and quantified as previously described (24). Polyclonal rabbit IgG containing 2.5 mg anti-BSA was instilled (in a volume of 300 μl) into the lungs via a tracheal cannula. The Ag, BSA (10 mg), was injected i.v. immediately thereafter in a volume of 0.5 ml. Rats were killed at the indicated times. Lung injury was quantified at 4 h by measuring increases in lung vascular permeability (extravascular accumulation of ^{125}I-labeled BSA). For blockade, either 300 μg preimmune rabbit IgG or anti-αd polyclonal rabbit IgG were instilled intratracheally with the anti-BSA. When hamster monoclonal IgG was employed, 200 μg hamster monoclonal anti-TNP IgG (Armenian hamster IgG clone G235–2356) or 200 μg hamster mAb to rat αd (clone 205c) was instilled with the anti-BSA.

Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109; and ICOS Corporation, Bothwell, WA 98021

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[2] Address correspondence and reprint requests to Dr. Peter A. Ward, Department of Pathology, The University of Michigan Medical School, M5240 Medical Science I, Box 0602, 1301 Catherine Road, Ann Arbor, Michigan 48109-0602, E-Mail: pward@umich.edu

[3] Abbreviations used in this paper: ICAM, intercellular adhesion molecule; BAL, bronchoalveolar lavage; CHO, Chinese hamster ovary; NO_2^-/NO_3^-, nitrite/nitrate; NFDM, nonfat dry milk; rαd/hulgG, recombinant rat αd "I" domain/human IgG; PBS-T, phosphate-buffered saline with 0.05% Tween-20; VCAM, vascular cell adhesion molecule.

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αd-specific polyclonal rabbit sera

Rabbit anti-rat αd polyclonal sera was generated to a recombinant rat αd "I" domain/human IgG (rαd/hulgG) fusion protein. The Ag was derived by subcloning cDNA from the "I" domain of rat αd (encompassing base pairs 469–1125) into an expression vector containing cDNA from the Fc region of human IgG4. The rαd/hulgG fusion protein was expressed in COS 7 cells and isolated from the cell supernatants by passage over a protein A column. The material was eluted with 0.1 M glycine buffer, pH 3.0, dialyzed against sterile PBS (pH 7.3), and found to be ~85% pure by SDS-PAGE analysis. Rabbits were initially immunized s.c. with the rαd/hulgG fusion protein emulsified in CFA, while subsequent boosts were administered using IFA. Ig from the anti-rat αd polyclonal rabbit sera was purified on a protein A column, then absorbed by passing it over a human IgG4 CNBr-Sepharose column to remove anti-human IgG reactivity. To test the specificity of the rabbit polyclonal sera, immunoprecipitations were done with rat spleen lysates. The rabbit polyclonal Ab was found to recognize only the rat αd/CD18 heterodimer (α-chain of ~145 kDa) and not the other β_2 integrins (see below).

Biotinylated cell lysates

Biotinylated bone marrow cell lysates were prepared from a Lewis rat. Briefly, femurs were excised and bone marrow cells were flushed from the bone with a 20-gauge needle and a 10-ml syringe containing PBS. The cells ($\sim 2 \times 10^8$) were labeled for 15 min at 25°C with 0.1 mg/ml normal human serum-sulfobiotin in 40 ml PBS followed by three consecutive washes with 50 ml PBS. The cells were pelleted and lysed in 2 ml lysis buffer (1% Nonidet P-40, 50 mM Tris (pH 8.0), 0.5 M NaCl, and 10 mM EDTA) containing 0.1 mM PMSF. Lysates were incubated 5 min at 25°C, vortexed for 30 s, then placed on ice for 15 min. The lysates were centrifuged to remove insoluble material.

Immunoprecipitation

Prior to the immunoprecipitation, 200 μl of protein A-Sepharose bead slurry (~1:2 beads to liquid) were added to 1 ml of cell lysates and mixed on an end-over-end rotator overnight at 4°C. The beads were pelleted and the precleared lysates aliquoted into 100-μl samples. For each immunoprecipitation, 10 μg of one of the following purified mAbs were added: (515F = mouse anti-rat CD11a (ICOS, Bothell, WA); OX42 = mouse anti-rat CD11b (Serotech, Raleigh, NC); 100G = hamster anti-rat CD11c (ICOS); 205C = hamster anti-rat αd (ICOS); 20C5B = mouse anti-rat CD18 (ICOS); and purified mouse IgG (Cappel, Durham, NC). The Ab was allowed to mix with the cell lysates on an end-over-end rotator for 2 h at 4°C.

To facilitate immunoprecipitations done with murine mAbs, protein A-Sepharose was armed with rabbit anti-mouse IgG. Approximately 0.5 ml protein A-Sepharose beads was mixed with 2 mg rabbit anti-mouse IgG and the slurry was allowed to mix for 30 min at 25°C. The protein A-Sepharose was washed and resuspended in 1 ml PBS. A total of 100 μl armed protein A-Sepharose slurry were added to each immunoprecipitation tube containing a mouse Ab. A total of 100 μl unarmed protein A-Sepharose were added to lysates containing hamster mAbs. The tubes were incubated end-over-end at 25°C for 30 min, the protein A-Sepharose was then pelleted and the cell lysates were removed. The protein-A Sepharose beads were washed three times in cold wash buffer (10 mM HEPES, 50 mM Tris (pH 8.0), 0.5 M NaCl, and 1% Triton X-100), resuspended in 20 μl 2 × SDS (containing 10% 2-ME) buffer, and boiled 5 min. The beads were pelleted and the liquid was run on a prepoured 8% SDS gel (Novex, San Diego, CA).

The protein was transferred to nitrocellulose and standard Western blot techniques were applied using a 1:10,000 dilution of Streptavidin-horseradish peroxidase (HRP) (Boehringer Mannheim, Indianapolis, IN) and the enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Rat αd transfectants

The rat αd cDNA was cloned from a rat spleen library, purchased from Clonetech Laboratories, (Palo Alto, CA). The library was screened under low stringency conditions using a 5′ probe generated from the human αd cDNA clone. A rat clone, designated 684.3, was identified in the screen, sequenced, and found to have 65% homology with the human αd cDNA.

DG44 CHO cells were cotransfected with a pDC1 plasmid containing full length rat αd and a pRC plasmid containing full length human CD18. The cells were plated onto a 150-cm tissue culture dish containing 20 ml culture medium (DMEM-F12, 10% FBS, 1 mM sodium phosphate, 2 mM L-glutamine, 100 U each of penicillin and streptomycin/ml, 0.1 mM hypoxanthine, and 0.016 mM thymidine). After 2 days the cells were transferred to selective media (DMEM-F12, 10% dialyzed FBS, 1 mM sodium phosphate, 2 mM L-glutamine, 100 U each of penicillin and streptomycin/ml, and 400 μg/ml G418/ml) and allowed to grow. When colonies were established, cells were split 1:2 every 3 to 5 days. Expression of a rat αd/huCD18 heterodimer was confirmed by FACS staining using the TS1/18.1 mAb (anti-human CD18) and the anti-rat αd polyclonal rabbit antisera. To increase the expression of the rat αd/CD18 complex, transfected CHO cells were subjected to several rounds of sorting of FACS using the rabbit anti-rat αd polyclonal antisera.

Generation of mAb to rat αd

Monoclonal antibody to rat αd was made in Armenian hamsters using the rαd/hulg fusion protein as the immunogen. The fusion wells were initially screened by FACS analysis using the αd/human CD18-transfected CHO cells. Positive fusion wells were subsequently screened for the ability to immunoprecipitate αd from biotin-labeled rat spleen lysates. The mAb, designated 205C, was identified in an early fusion and further characterized. To verify the specificity of the 205C mAb, rat spleen lysates were first precleared of all other α-chains of the β_2 integrin family, including CD11a, CD11b, and CD11c, by immunoprecipitation. Following this step, 205C continued to immunoprecipitate a 145 kDa/95 kDa heterodimer consistent with the known size of the αd/CD18 complex. To complete the characterization, the mAb was used to affinity purify αd from rat splenocyte lysates. The N-terminal sequence analysis from the affinity-purified protein was found to be consistent with amino acid sequence predicted by the αd cDNA clone (data not shown).

In vitro production of NO_2^-/NO_3^-

Rat alveolar macrophages were recovered as previously described (27). Cells (1×10^5/well) were cultured in 96-well tissue culture plates in DMEM and nonadherent cells removed following 1-h incubation at 37°C in 7.5% CO_2 in air. Cells were stimulated with murine IFN-γ (25 U/ml) and 10 μg bacterial LPS at 37°C for 18 h and supernatant fluids collected and analyzed for NO_2^-/NO_3^-.

Immunostaining techniques

For immunostaining of frozen sections, lungs from injured rats were frozen in OCT compound (Miles Co., Elkhart, IN) and stained with hamster mAb 205C diluted 1:1000 in PBS containing 0.1% BSA for 1 h in a humidified chamber. Slides were then washed two times in PBS and incubated for 1 h with HRP-conjugated rabbit anti-hamster IgG-specific Ab (Rockland, Gilbertsville, IL) diluted 1:10,000 in PBS. Slides were washed two times in PBS, dried, and incubated with HRP-specific substrate True Blue (Kirkegard & Perry, Gaithersburg, MD) for 5 min. Slides were then dipped in 100% ethanol. When BAL macrophages were stained for rat αd, cytospin preparations were used employing similar immunostaining methods.

Western blot analysis

Lung homogenates were prepared from rats undergoing IgG immune complex-induced lung injury at times 0, 1, 2, and 4 h and separated by electrophoresis on SDS-polyacrylamide gels (15%). Homogenate protein levels were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). A total of 100 μg obtained from each time point were loaded per lane under nonreducing conditions. Separated proteins were transblotted to polyvinylidene difluoride membrane (Westran, Schleicher and Schuell, Keene, NH) for 1 h at 10 V. The membrane was blocked overnight at 4°C with 5% nonfat dry milk (NFDM) in PBS and then washed three times with 0.05% Tween-20 in PBS (PBS-T). The membrane was incubated for 1 h at room temperature with primary Ab (rabbit anti-mouse αd) at a dilution of 1/100 in 1% NFDM in PBS. After washing three times in PBS-T, secondary Ab (goat anti-rabbit IgG HRP-conjugated Ab; Bio-Rad) was added at a final dilution of 1/10,000 in 1% NFDM-PBS and incubated for 1 h at room temperature. After washing, the membrane was developed by enhanced chemiluminescence technique according to the manufacturer's protocol (Amersham Co., Little Chalfont, U.K.).

Northern blot analysis

Following IgG immune complex deposition, rats were sacrificed at 2-h intervals from 0 to 4 h. Whole lungs were dissected and frozen in liquid nitrogen for Northern blot analysis of IL-6 mRNA. RNA was extracted using a guanidinium-isothiocyanate method as described previously (27, 28). Twelve micrograms of cytoplasmic RNA were fractionated electrophoretically in a 1% formaldehyde gel and transferred to a nylon blot (Zetabind, CUNO Laboratories, Meriden, Ct). Equal loading of samples was confirmed by methylene blue staining of 18S and 28S rRNA bands. Rat αd cDNA clone was used as a template to generate a PCR probe encompassing base pairs 2014–2871. The region of the gene was chosen

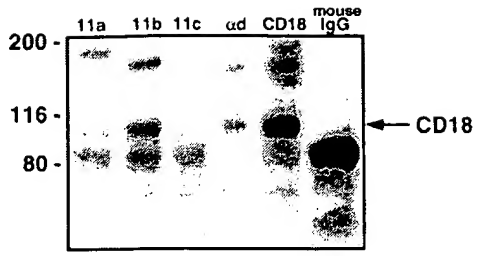

FIGURE 1. Immunoprecipitation products from rat bone marrow cells using anti-rat Abs to: CD11a (5I5F), CD11b (0 × 42), CD11c (100G), αd (205C), CD18 (20C5B), and mouse IgG. Bands were developed by Western blot analysis after electrophoresis of samples in 8% SDS gels under reducing conditions.

for lack of homology to the other integrin α-chains. The cDNA for rat αd was [^{32}P]dCTP-radiolabeled (NEN-DuPont, Boston, MA) by PCR to generate the cDNA probe that was applied to the Northern blot. Hybridization was performed at 65°C for 18 h and the autoradiogram was developed on Kodak X-Omat film.

For 18S band labeling, the Northern blot was prehybridized for 4 h on 6× SSC with 1% SDS. An oligomer for the 18S band (5′-GACAAG CATATGCTACTGGC-3′) was labeled with [γ-^{32}P]ATP (10 pmol) using T4 polynucleotide kinase (10 U, Life Technologies) incubated for 30 min at 37°C. A second aliquot of kinase (10 U) was added and the reaction was incubated an additional 15 min. The reaction was terminated with 2 ml 0.5 M EDTA. Unincorporated [γ-^{32}P]ATP was removed by spin-column chromatography (BioSpin 6 column, BioRad). Labeled probe was combined with 8 mg of unlabeled oligonucleotide and heated for 1 min at 95°C prior to hybridization with the Northern blot. The blot was incubated for 18 h at 42°C. Following hybridization, the blot was washed twice with 3× SSC with 1% SDS for 15 min each at 50°C and developed using a phosphor imaging screen.

BAL fluid neutrophil counts and TNF-α content

BAL fluids were collected from rats sacrificed at 4 h following commencement of injury by instilling and withdrawing 9 ml sterile Dulbecco's PBS (without Ca/Mg) three times from the lungs via an intratracheal cannula. Total white cell counts were determined using a Coulter counter (Coulter Electronics, Hialeah, FL). Specimens for cell differentials were prepared using cytospin centrifugation (700 × g for 7 min) on BAL fluids. Specimens were fixed and stained with Diff-Quik products (Baxter Co., Miami, FL) for determination of percentage of neutrophils and macrophages/monocytes. The total numbers of neutrophils for each BAL sample were then determined according to the volume of BAL recovered. Remaining BAL samples were centrifuged at 1500 × *g* for 10 min and the supernatant fluids frozen and subsequently evaluated for TNF-α activity using a standard WEHI cell cytotoxicity assay as previously reported (29). Time course studies of TNF-α expression have shown 4 h to be a time point of peak expression.

Measurement of NO_2^-/NO_3^- in BAL fluids and culture supernatant fluids

Nitrite was measured with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 25% hydrochloric acid) which forms a chromophore absorbing at 543 nm (27). Any nitrate present was reduced to nitrite with nitrate reductase (EC 1.6.2.2) from *Aspergillus* sp., to which 2.5 nM NADPH was added. Absorbance was then measured as NO_2^- (the combination of nitrite and reduced nitrate being designated as NO_2^-/NO_3^-). These measurements were presented as nanomoles per milliliter of BAL fluid or nanomoles in culture supernatant fluids.

Statistical analysis

All values were expressed as mean ± SEM. All statistical comparisons were made between treatment groups and positive controls after values obtained from negative controls had been subtracted from each data point. Two-way analysis of variance was determined, together with use of the Scheffe *t* test and the Protected Least Significant Difference test. Statistical significance was defined as $p < 0.05$.

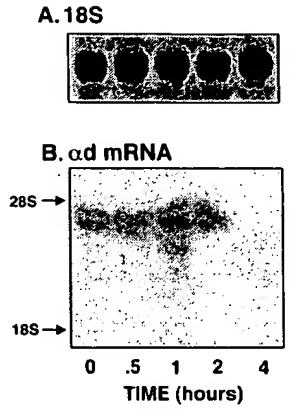

FIGURE 2. Northern blot analysis for whole lung mRNA for rat αd at 0, 1, 2, and 4 h after induction of lung injury. Data for gel loading are also shown.

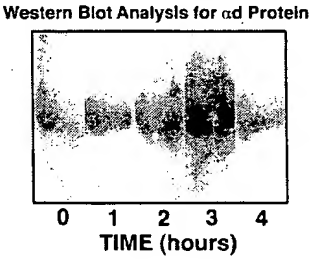

FIGURE 3. Western blot analysis for rat αd in homogenates obtained at 0, 1, 2, 3, and 4 h after intrapulmonary deposition of IgG immune complexes.

Results

Immunoprecipitation of rat CD11 proteins

Rat bone marrow cells were biotinylated and subjected to immunoprecipitation techniques described above. The results, appearing as Western blots and using mAb (205c), are shown in Figure 1. In each case, bands consistent with the m.w. of CD18 appeared in the region between the marker positions of 85 and 115 kDa. Bands representing the higher m.w. α-chain proteins were present, in the region between 130 and 180 kDa. In the case of αd, a band in the position of CD18 was found, together with a higher m.w. protein of ~145 kDa. The reference murine IgG demonstrated the expected heavy and light chains.

Expression of αd mRNA and αd protein in inflamed rat lung

Lungs from animals undergoing intrapulmonary deposition of IgG immune complexes were extracted for RNA and evaluated by Northern blot analysis for αd mRNA and by Western blot for αd protein. The results of these studies are shown in Figures 2 and 3. Faint, constitutive expression of mRNA could be detected in lung at time 0, but increases in mRNA were found between 0.5 and 2.0 h. Thereafter, a progressive reduction in αd mRNA was found. In the *upper part* of Figure 2, equal loading of RNA was confirmed by the use on oligonucleotide that detects 18S ribosomal RNA. As shown in Figure 3, using Western blot analyses, homogenates from immune complex-injured lungs at 0, 1, 2, 3, and 4 h revealed slight constitutive αd (at 0 h) and clear and increasing amounts of αd, peaking at 3 h, followed by a reduction at 4 h. These findings indicate that IgG immune complex-induced lung inflammation in rats causes up-regulation of lung mRNA and protein for αd in a time-dependent manner, but that the up-regulation is not sustained.

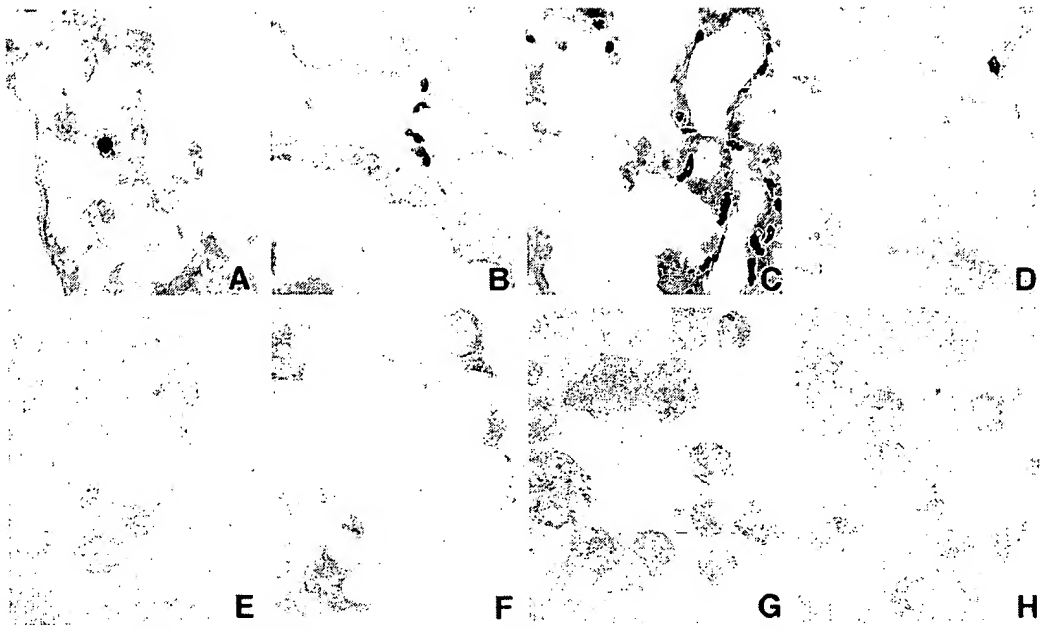

FIGURE 4. Immunostaining for αd in frozen sections of lung at 0, 1, 2, and 4 h after deposition of IgG immune complexes (*A–D*, respectively). Also displayed are alveolar macrophages obtained by BAL procedures at 0, 1, 2, and 4 h and stained for αd protein (*lower frames*).

Immunostaining of lung cells and tissue for αd

Frozen sections of rat lungs were obtained at 0, 1, 2, and 4 h after intrapulmonary deposition of IgG immune complexes and stained for αd. As shown in Figure 4 (*A–D*), little reaction product (appearing as a brown stain) was found in lungs at 0 h (*A*). By 1 h (*B*), more reaction product was found. It was markedly accentuated at 2 h (*C*) and appeared in interstitial areas and along alveolar surfaces. By 4 h (*D*), there was a dramatic reduction. Alveolar macrophages were retrieved by BAL procedures at the same time points and stained for αd. Very little reaction product was found at 0 time (*E*). At 1 and 2 h, positive staining of alveolar macrophages could be seen (*F* and *G*, respectively). By 4 h (*H*) very little reaction product was found, consistent with the immunostaining of lung sections (*A–D*) and the Western blot (Fig. 3).

Effects of Ab to αd on lung injury and lung content of neutrophils and TNF-α

Rats undergoing IgG immune complex-induced lung inflammation were evaluated for the ability to block rabbit polyclonal Ab to rat αd to affect the outcome of lung inflammatory reactions. In data shown in Figure 5*A*, the positive control groups received, together with the intratracheally administered anti-BSA, either 300 μg preimmune rabbit IgG or 300 μg rabbit IgG anti-αd. The effect on pulmonary vascular permeability (as measured by albumin leak into lung) was then determined 4 h after initiation of the reactions. The differences in the permeability indices in the negative and positive (treated with preimmune IgG) controls were approximately fivefold. In the presence of polyclonal anti-αd, there was a 55% reduction ($p < 0.001$) in the permeability index when compared with the positive control group treated with 300 μg preimmune IgG. In a companion set of animals, neutrophils retrieved by BAL were quantitated as a function of treatment (Fig. 5*B*). When compared with negative controls (receiving anti-BSA only with omission of the i.v. administration of Ag), positive controls that received 300 μg preimmune IgG intratracheally together with anti-BSA showed a sixfold increase in numbers of BAL neutrophils,

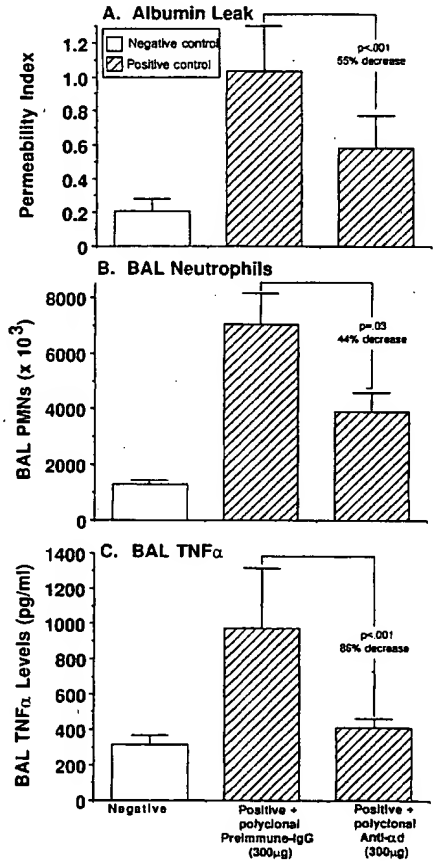

FIGURE 5. Effects of 300 μg polyclonal rabbit preimmune IgG or anti-rat αd IgG on intrapulmonary [^{125}I]albumin leak (*A*), lung content of MPO (*B*), and BAL fluid levels of TNF-α (*C*) 4 h after induction of IgG immune complex-mediated lung injury. For each vertical bar, $n = 5$.

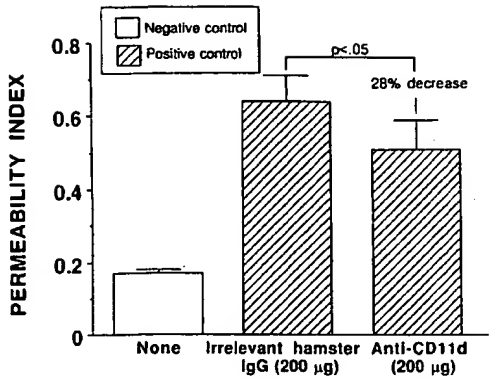

FIGURE 6. Protective effects of 200-μg hamster monoclonal anti-rat αd on IgG immune complex-induced lung injury, as revealed by albumin leak into lungs. Reference positive controls received irrelevant hamster IgG. For each vertical bar, $n = 6$.

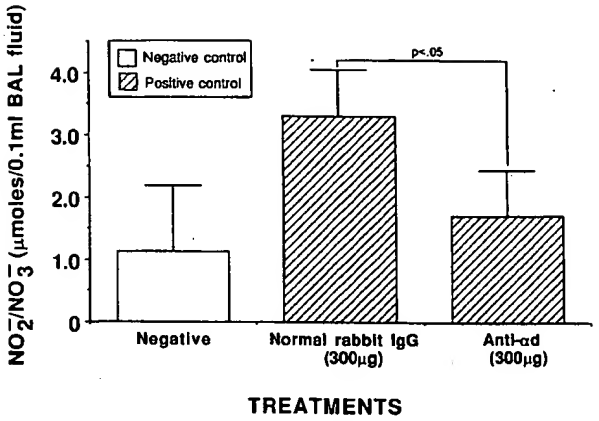

FIGURE 7. Effects of treatment with 300 μg polyclonal rabbit anti-rat αd on NO_2^-/NO_3^- levels in BAL fluids obtained 4 h after induction of lung inflammatory reactions. The positive control groups received 300 μg normal rabbit IgG or 300 μg rabbit IgG anti-αd together with the anti-BSA. Details of the protocol are similar to those described in Figure 2. For each vertical bar, $n = 4$.

rising from $1.28 \pm 0.14 \times 10^6$ in the negative controls to $7.04 \pm 1.09 \times 10^6$ in the positive controls ($p < 0.001$). In rats receiving an intratracheal instillation of 300 μg IgG anti-αd together with the anti-BSA, the yield of neutrophils fell by 44% ($p = 0.03$), to $3.92 \pm 0.672 \times 10^6$.

Finally, the same BAL fluids were evaluated for TNF-α, a cytokine that is required for up-regulation of lung vascular ICAM-1 and E-selectin adhesion molecules, which are essential for recruitment of neutrophils (24, 29, 30). Under the same experimental conditions described above, negative controls had BAL TNF-α levels of 316 ± 51 pg/ml, while positive controls receiving 300 μg preimmune rabbit IgG together with the anti-BSA had 973 ± 33 pg TNF-α/ml (Fig. 5C). In contrast, the presence of anti-αd in the anti-BSA preparation dramatically reduced (86%, $p < 0.001$) the rise in TNF-α levels (to 409 ± 56 pg TNF-α/ml). These data indicate that in the experimental lung inflammatory model employed αd plays an important role in neutrophil recruitment and development of lung damage, in part by facilitating lung expression of TNF-α.

A companion series of studies was undertaken to assess the effects of hamster mAb to rat αd. The experimental protocol was similar to that described in Figure 5A. Negative controls received anti-BSA intratracheally while positive controls received anti-BSA together with 200 μg hamster irrelevant IgG mAb to trinitrophenol. The other group of positive control animals received 200 μg hamster mAb (205C) to rat αd, which was instilled intratracheally together with the anti-BSA. Limitations in amounts of available mAb precluded experiments with higher doses. The results are shown in Figure 6. In this experiment, the permeability rise in the positive control group, as compared with the negative control group, was approximately fourfold (rising from a value of 0.17 ± 0.01 to a value of 0.64 ± 0.007). In the presence of mAb to rat αd, the permeability value fell by 28% ($p < 0.05$), to a value of 0.51 ± 0.08. Thus, blockade of rat αd by either polyclonal or monoclonal antibody is protective in this model of lung injury, as reflected by albumin leak.

Effects of anti-αd on BAL fluid levels of NO_2^-/NO_3^-

In rats undergoing IgG immune complex deposition, BAL fluids were obtained at 4 h and evaluated for NO_2^-/NO_3^- content. The groups included negative controls, which received anti-BSA intratracheally in the absence of BSA, and positive controls, which received 300 μg preimmune rabbit IgG or 300 μg of rabbit anti-rat αd IgG intratracheally together with the anti-BSA. The results are shown in Figure 7. BAL fluids from negative controls contained low levels of NO_2^-/NO_3^- (1.63 ± 1.49 nmol/ml), rising nearly threefold, to 3.29 ± 0.75 nmol/ml in positive controls treated with preimmune rabbit IgG. In the presence of anti-rat αd, the increased production of NO_2^-/NO_3^- was significantly suppressed ($p < 0.05$) in BAL fluids (falling to 1.71 ± 0.74 nmol/ml), indicating that αd is required for full lung production of NO, an intermediate known to be involved in development of lung injury in this model (31).

In vitro inhibition of NO_2^-/NO_3^- production by stimulated macrophage

Experiments were done to determine if the Ab to αd would affect NO_2^-/NO_3^- production in rat alveolar macrophages that had been stimulated with LPS (10 μg/ml) and murine IFN-γ (25 U/ml) at 37°C for 18 h. These experiments were designed after preliminary observations suggesting that αd on rat macrophages was expressed after incubation with LPS and IFN-γ (R. L. Warner and P. A. Ward, unpublished observations). Supernatant fluids were collected at the end of the incubation period and analyzed for NO_2^-/NO_3^-. In parallel sets of wells, increasing amounts (from 0 to 50 μg/ml) of rabbit polyclonal IgG Ab to rat αd were added at time 0. Other controls included normal rabbit IgG at the concentration of 50 μg/ml. The results are shown in Figure 8 where anti-rat αd caused a progressive reduction in NO_2^-/NO_3^- formation as a function of the Ig concentration. A plateau appeared to be reached when the concentration of Ab was 10 μg/ml. The maximal amount of inhibition of NO_2^-/NO_3^- production induced by anti-αd was 63%. Irrelevant rabbit IgG had no statistically significant effect on generation of NO_2^-/NO_3^-.

Discussion

Expression of αd by normal cells and normal tissues appears to be highly restricted. In normal tissues, αd is expressed primarily by macrophages in the splenic red pulp, cells in the bone marrow and in medullary regions of lymph nodes, while in the blood αd expression is limited to a small subset of $CD8^+$ cells (32). One of the counter-receptors identified for human αd is human ICAM-3, a member of the Ig superfamily. Although intensive efforts have been made, neither mouse nor rat ICAM-3 has been cloned (W. M.

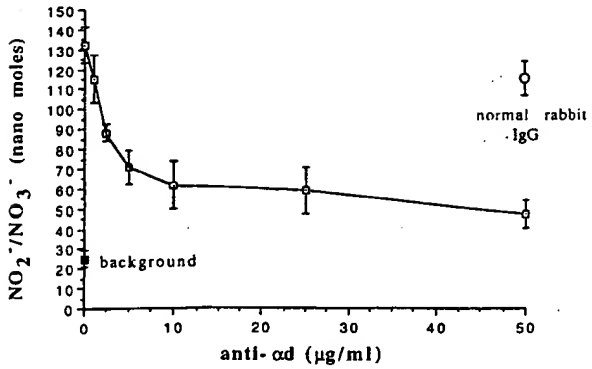

FIGURE 8. Dose-response inhibition of NO_2^-/NO_3^- production in rat alveolar macrophages stimulated with LPS and IFN-γ. Increasing amounts of rabbit anti-rat αd were added to wells containing macrophages at time 0. NO_2^-/NO_3^- generation was determined after 18 h in culture.

Gallatin, personal communication). Although it seems likely that rodents contain an ICAM-3 homologue (in view of the importance of ICAM-3 in activation of human T cells) (7), the situation could be analogous to the absence of IL-8 in rats or mice. Alternatively, it is possible that there exists in rats (and mice) another counter-receptor for αd. There are suggestions that human αd is reactive with VCAM-1 (W. M. Gallatin and M. Van der Vieren, personal communication). Whether rat VCAM-1 may function as a counter-receptor for rat αd remains to be determined. It seems likely that αd/CD18, like other β_2 integrins, interacts with ICAM molecules, serves as an activation marker, and participates in signal transduction events in stimulated lymphocytes, monocytes, and macrophages. It has previously been shown that full expression of lung injury in the IgG immune complex model in rats requires both LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (33).

We have recently suggested that alveolar macrophages may be in adhesive contact with ICAM-1 in surfaces of alveolar type II cells, the result of which permits macrophages to optimally produce TNF-α stimulated with IgG immune complexes and complement activation products (34). This conclusion is also based on the finding that intratracheally administered blocking Ab to rat ICAM-1 suppresses lung inflammatory injury in the same inflammatory model as described in this paper. A key role for C5a has recently been shown in the IgG immune complex model, since blockade of C5a by polyclonal Ab to rat C5a suppresses inflammation and, surprisingly, substantially reduces up-regulation of lung vascular ICAM-1. This outcome has been found to correlate with greatly reduced levels of BAL fluid levels of TNF-α (35).

The current studies extend knowledge regarding the roles of adhesion molecules involved in neutrophil recruitment in the IgG immune complex-induced injury model. As indicated above, both LFA-1 and Mac-1 are involved in this model of injury, with LFA-1 being tied to neutrophil recruitment events in the vascular compartment and Mac-1 being involved in events (TNF-α production) taking place in the distal airway compartment of the lung (33, 34). LFA-1 appears to be directly involved in neutrophil adhesion to the vascular endothelium, probably through neutrophil LFA-1 interaction with up-regulated vascular ICAM-1. A driving force for vascular ICAM-1 up-regulation appears to be TNF-α derived from lung macrophages. The role of Mac-1 may be related to alveolar macrophage Mac-1 interacting with alveolar epithelial cell ICAM-1, resulting in maximal protection by adherent macrophages of TNF-α. Airway blockade of either Mac-1 (but not LFA-1) or ICAM-1 reduces TNF-α production, neutrophil recruitment, and lung damage (34). We have also been shown that mAb to rat VLA-4 is protective in the same model of lung injury, interfering with full recruitment of neutrophils (33). Since rat neutrophils contain little, if any, detectable VLA-4 (R. S. Warner and P. A. Ward, unpublished observations), it is possible that lung macrophage VLA-4 is reactive with macrophage VCAM-1 and that this adhesion-promoting process facilitates optimal production of cytokines by macrophages. The presence of VCAM-1 in macrophages has been reported (36, 37). As described above, it is also possible (but not demonstrated) that rat αd is reactive with rat VCAM-1. The role of rat αd in the IgG immune complex model of lung injury suggests that, like ICAM-1, it is required for full production of TNF-α by lung macrophages, leading to up-regulation of lung vascular ICAM-1 and neutrophil recruitment. The fact that airway instillation of anti-rat αd reduced BAL TNF-α levels by 86% (Fig. 5) is consistent with this possibility. What is unclear is the counter-receptor in rat lung for rat αd.

The data in this report indicate that the full recruitment of neutrophils and expression of lung injury after deposition of IgG immune complexes requires αd which, like CD11b, plays an important role in the full expression of macrophage-generated TNF-α, a critical molecule involved in vivo in the up-regulation of endothelial ICAM-1. Additional in vivo roles of αd remain to be determined. Thus, in the IgG immune complex-induced model of lung injury, lung macrophage αd, like CD11b/CD18, facilitates the full production of TNF-α, which induces vascular ICAM-1 and E-selectin up-regulation and subsequent neutrophil recruitment. It also appears that macrophage αd subserves another proinflammatory function, namely, facilitating production of NO, as indicated by measurements of NO_2^-/NO_3^-. As suggested above, both TNF-α and NO play important lung-damaging functions, the former by facilitating neutrophil recruitment and the latter (or its derivatives) by causing direct damage to lung cells and extracellular matrix.

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